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The adenomatous polyposis coli (*APC*) gene is considered to be a gatekeeper in colorectal tumourigenesis. Inactivating mutations in *APC* have been reported in 34–70% of sporadic colorectal cancer patients, the majority of which occur in the mutation cluster region (MCR). In this study, tumour tissue from 665 incident colorectal cancer patients, who originate from 120 852 men and women (55–69 years of age at baseline) participating in The Netherlands Cohort Study, was evaluated for the occurrence and type of *APC* mutations with regard to age at diagnosis, gender, family history of colorectal cancer, Dukes' stage, tumour differentiation and sub-localization. Mutation analysis of the MCR, which spans codons 1286–1513, was performed on archival adenocarcinoma samples using macrodissection, nested PCR and direct sequencing of purified PCR fragments. A large number of genetic aberrations ($n = 978$), including point mutations ($n = 833$), deletions ($n = 126$) and insertions ($n = 19$) was detected in the MCR in 72% of patients (479/665). In particular, we observed a large number of missense mutations, more than reported previously. This may indicate involvement in colorectal carcinogenesis, although their significance for APC functions is unclear. Truncating mutations were found in 37% of patients (248/665). Patients with rectosigmoid and rectum tumours relatively more frequently harboured C > T nonsense mutations and truncating frameshift mutations as compared with patients with proximal and distal colon tumours ($P = 0.009$ and $P = 0.045$, respectively). Differences in occurrence of truncating mutations with regard to tumour sublocalization suggest a different aetiology of tumourigenesis in colon and rectum.

Introduction

It is well established that sporadic colorectal cancer (CRC) arises through a gradual series of histological changes, which are accompanied by specific genetic alterations involving tumour suppressor genes (e.g. *APC*, *DCC*, *SMAD4*, *TP53*) and oncogenes (e.g. *K-ras*) (1,2). Adenomatous polyposis coli (*APC*) gene mutation is considered to be an early event in colorectal carcinogenesis.

Linkage analysis of families with the autosomal dominant inherited disease familial adenomatous polyposis coli (FAP) has led to mapping of the *APC* gene to 5q21 (3). This large gene with 21 exons, was subsequently cloned, identified and characterized (4). Mutations in both *APC* alleles were also found in most sporadic colorectal tumours (5). Whereas germline mutations in FAP are confined to the 5′ half of the *APC* gene, somatic mutations are clustered in the central region of the open reading frame, spanning codons 1286–1513 of exon 15 (5,6). Although this mutation cluster region (MCR) represents only 8% of the 8535 bp *APC* gene, 68–77% of somatic mutations in *APC* occur in this region (7,8).

The APC protein consists of 2843 aa, and comprises a number of functional domains, motifs and other signatures, as reviewed by Polakis (6). APC is capable of binding β-catenin through its 15 aa repeats, situated between aa 1020 and 1169 (9). More importantly, in the central region of APC, seven motifs of 20 aa were identified, which not only bind β-catenin but also facilitate down-regulation of β-catenin through interactions with Axin and GSK3β (10,11). Through inactivating mutations in the central part of the *APC* gene, which often lead to complete or partial loss of function of this region, β-catenin levels are up-regulated and the Wnt signalling pathway is activated, which in turn may lead to enhanced proliferation (12). In addition to its role in the Wnt pathway, APC has other functions. Through its binding of β-catenin APC is also linked to E-cadherin and α-catenin, which in turn bind actin and actin-associated proteins, thus taking part in intercellular adhesion (13). Additionally, APC associates with the microtubule cytoskeleton directly through its C-terminus. There is also evidence that APC may play a role in the regulation of apoptosis (14).

Frequencies and specific types of somatic mutations in the *APC* gene have been investigated in a number of studies. Somatic mutations in *APC* have been found in 34–70% of sporadic tumours in CRC patients (7,8,15–21). Higher prevalences of 75–95% have been found in studies on the occurrence of somatic *APC* mutations in CRC cell lines (22–24). The large majority of mutations observed in different studies would lead to a truncated and therefore inactivated APC protein, either by nonsense point mutations (30%) or by frameshift mutations (68%), as reviewed by Béroud (25). Most reports, however, were based on limited numbers of (usually selected) patients. Studies on somatic *APC* mutations are often based on FAP patients and it has been shown that the occurrence of a second mutation in the *APC* gene is somehow dependent on the position of the germline mutation, thereby introducing a bias (26). Examination of *APC* mutations in colorectal cell lines has indicated that there may also be an interdependence of

Abbreviations: APC, adenomatous polyposis coli; CRC, colorectal cancer; FAP, familial adenomatous polyposis coli; MCR, mutation cluster region.

the two hits in *APC* in sporadic colorectal cancer (23). Furthermore, results from different reports are difficult to compare as different methods of detecting mutations in *APC* have been used and different regions of the *APC* gene have been studied.

In the current report, the frequency and type of *APC* gene mutations are assessed in a large series of unselected, incident CRC patients from The Netherlands identified in a prospective cohort study. The occurrence and type of *APC* gene mutations in the MCR are reported with regard to age at diagnosis, gender, family history of colorectal cancer, Dukes' stage, tumour differentiation and sub-localization.

Materials and methods

Study population

A prospective cohort study on diet and cancer has been initiated in The Netherlands in September 1986. The study design has been described in detail elsewhere (27). Briefly, at baseline a total of 58 279 men and 62 573 women, between the ages of 55 and 69 years, completed a self-administered food frequency and lifestyle questionnaire. The study population originated from 204 municipal population registries throughout the country. Incident cancer cases are identified by monitoring of the entire cohort for cancer occurrence through annual record linkage to The Netherlands Cancer Registry (NCR), nine regional cancer registries throughout The Netherlands, and to the 'Pathologisch Anatomisch Landelijk Geautomatiseerd Archief' (PALGA), a nationwide database of pathology reports (28). The PALGA database was used to identify and locate tumour tissue in Dutch Pathology laboratories. The first 2.3 years of follow up were excluded due to incomplete nationwide coverage of PALGA. From 1989 until 1994, 819 incident cases with histologically confirmed CRC were identified. CRC was classified according to site as follows, proximal colon: cecum through transverse colon (ICD-O codes 153.0, 153.1, 153.4, 153.5, 153.6); distal colon: splenic flexure through sigmoid colon (ICD-O codes 153.2, 153.3, 153.7); rectosigmoid (ICD-O code 154.0) and rectum (ICD-O code 154.1). Information about age at diagnosis, gender and family history of CRC (at baseline) was retrieved from The Netherlands Cohort Study on diet and cancer (NLCS) database. Information about tumour sub-localization, Dukes' stage and differentiation was retrieved from the NCR database.

Tissue samples

Tumour material of CRC patients was collected after approval by the Ethical Review Board of University Maastricht, PALGA and the NCR. Tissue samples from 819 CRC patients were retrieved from 54 pathology laboratories throughout The Netherlands. Tumour tissue specimen collection started in August 1999 and was completed in December of 2001. Forty-four tumour tissue samples (5%) could not be traced. Of 775 eligible tissue samples 737 (90%) contained sufficient tumour material as confirmed by a pathologist (A.d.B.) for molecular analyses.

DNA isolation

DNA isolation was described in detail elsewhere (29). Briefly, one 5 μm section was taken from each paraffin-embedded tumour tissue block and stained with haematoxylin and eosin for histopathological examination. Five 20 μm sections of tumour tissue were taken from each sample for DNA isolation. After deparaffination of the sections, tumour tissue was separated from the normal colonic epithelium using the haematoxylin and eosin section as a reference. Genomic DNA was extracted from macrodissected tumour tissue using proteinase K (Qiagen, St Louis, MO) and the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). DNA concentration and purity was measured in a spectrophotometer at 260 and 280 nm. DNA from the fresh, unfixed CRC cell lines and the 10 fresh-frozen tissue samples was extracted as described for paraffin-embedded sections.

APC mutation analysis

Since the majority of somatic mutations in APC occur within the MCR, we amplified the MCR as four overlapping fragments (codons 1286–1520) in a nested PCR strategy. Flank PCR was performed to generate two fragments A and B. Genomic DNA (250 ng) was added to 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 2.0 mM $MgCl_2$, 250 μM dNTP (Pharmacia, Uppsala, Sweden), 200 nM of each primer (Table I), and 1 U Platinum *Taq* (Invitrogen, Breda, The Netherlands) in a final volume of 50 μl. Fragment A was used as starting material for the amplification of nested fragments S1 and S2, and fragment B was used for nested fragments S3 and S4. An alternative semi-nested PCR strategy was chosen when nested PCR amplification failed for one or more fragments originating from flank A or B. Alternative flank fragments F1 to F4 were then generated for each of the four nested fragments S1 to S4, respectively. The (semi-)nested PCR was performed in a final volume of 50 μl, containing 5 μl of a 1:100 dilution of the flank product, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5–2.5 mM $MgCl_2$, 250 mM dNTP, 200 nM of each primer (Table I), and 1 U Platinum *Taq*. The four (semi-)nested fragments were labelled with biotin at one of the two primers for subsequent direct sequencing as indicated in Table I. The cycles for both flank, nested and

Table I. Fragment characteristics for the amplification and sequencing of the MCR of APC

Fragment	Nucleotide position	Fragment size (bp)	Primer sequences
Flank A	3874–4229	356	Sense: 5′-GAA ATA GGA TGT AAT CAG ACG-3′ Antisense: 5′-GA GCT GGC AAT CGA ACG ACT-3′
Flank B	4114–4624	511	Sense: 5′-GCT CAG ACA CCC AAA AGT CC-3′ Antisense: 5′-C ATT CCC ATT GTC ATT TTC C-3′
Flank F1	3796–4092	297	Sense: 5′-CAG ACT TAT TGT GTA GAA GA-3′ Antisense: 5′-CGC TCC TGA AGA AAA TTC AAC-3′
Flank F2	3982–4003	248	Sense: 5′-GAA GTT CCA GCA GTG TCA CAG C-3′ Antisense: 5′- GA GCT GGC AAT CGA ACG ACT-3′
Flank F3	4114–4383	270	Sense: 5′-GCT CAG ACA CCC AAA AGT CC-3′ Antisense: 5′-ATT TTT AGG TAC TTC TCG CTT G-3′
Flank F4	4328–4624	297	Sense: 5′-AA ACA CCT CCA CCA CCT CC-3′ Antisense: 5′-C ATT CCC ATT GTC ATT TTC C-3′
Nested S1	3874–4092	219	Sense: 5′-GAA ATA GGA TGT AAT CAG ACG-3′[a] Antisense:5′-CGC TCC TGA AGA AAA TTC AAC-3′ Sequence primer: 5′ (Cy5)-CGC TCC TGA AGA AAA TTC AAC AGC-3′
Nested S2	4026–4229	204	Sense: 5′-A CTG CAG GGT TCT AGT TTA TC-3′[a] Antisense: 5′-GA GCT GGC AAT CGA ACG ACT-3′ Sequence primer: 5′ (Cy5)-GA GCT GGC AAT CGA ACG ACT CTC-3′
Nested S3	4179–4383	205	Sense: 5′-T ACT TCT GTC AGT TCA CTT GAT A-3′[a] Antisense: 5′-ATT TTT AGG TAC TTC TCG CTT G-3′ Sequence primer: 5′ (Cy5)-ATT TTT AGG TAC TTC TCG CTT GGT TTG-3′
Nested S4	4328–4594	267	Sense: 5′-AA ACA CCT CCA CCA CCT CC-3′ Antisense: 5′-G CAT TAT TCT TAA TTC CAC ATC-3′[a] Sequence primer: 5′ (Cy5)-AA ACA CCT CCA CCA CCT CCT CA-3′

[a]Biotin-labelled primer.

semi-nested PCR consisted of 3 min pre-denaturation at 94°C, 40 s at 94°C for denaturation, 1 min at 52°C to 57°C for annealing, 1 min at 72°C for elongation and 10 min at 72°C for post-elongation. In each PCR, one round of 35 cycles was performed using a Peltier Thermal Cycler-200 (MJ Research, Biozym, Landgraaf, The Netherlands) or a I-cycler (BioRad, Veenendaal, The Netherlands). In each PCR series positive (DNA from CRC cell lines) and negative controls (no DNA) were included. The length and concentration of the PCR products were checked by electrophoresis on 2% agarose gels and visualized with ethidium bromide.

Mutation analysis was performed by direct sequencing using the Autoload SPS Sequencing Kit according to the manufacturer's instructions (Amersham Biosciences, Roosendaal, The Netherlands). In brief, the biotinylated PCR product was captured on a sequencing comb coated with streptavidin. After removal of the non-biotinylated strands by alkaline denaturation, the remaining immobilized strand was used as a template for dideoxy sequencing reactions with extended Cy5 labelled primers (Table I) and T7 DNA polymerase. The sequence profile was analysed on ALFexpress DNA Analysis System using ALFwin software (Amersham Biosciences, Roosendaal, The Netherlands). Evaluation of the sequence patterns was performed by two independent observers, based on the criteria that an increase of at least 5% is observed for the mutant peak, as well as a decrease of at least 5% of the wild-type peak, relative to the wild-type pattern in the same sequence run. The reference sequence for human *APC* was derived from Genbank Accession Number M74088.1. Data entry was performed blindly, by two independent observers.

From 72 of the 737 patients whose tumour samples contained sufficient DNA, one or more fragments of the MCR could not be amplified and these patients were not included in this study. The 665 patients, from whom MCR mutation analysis was complete, were comparable with the 819 CRC patients initially recognized within the cohort with respect to mean age at diagnosis, gender, family history of CRC, Dukes' stage, tumour differentiation and sub-localization.

Validity and reproducibility of APC mutation analysis

In order to validate mutation analysis on paraffin-embedded tissue, 10 freshly obtained CRC specimens were each divided into two adjacent tissue blocks, one of which was fresh-frozen, and the other routinely fixed and embedded in paraffin. Two samples contained wild-type *APC*, three samples harboured a mutation and five samples had a polymorphism. These control specimens were obtained from patients who did not participate in the NLCS. Six CRC cell lines, i.e. HT29, Colo205, CaCo2, SW480, HCT116 and LOVO (obtained from the American Type Culture Collection, Rockville, MD) were used to check the specificity of mutation detection in the MCR of *APC*. The detection limit of mutation analysis was determined by mixing homozygously mutated DNA isolated from CaCo2 cells (C > T substitution at codon 1367) with increasing concentrations of the corresponding wild-type DNA sequence prepared from SW480 cells. Reproducibility of mutation analysis was established by subjecting 72 fragments, derived from 54 NLCS adenocarcinoma specimens twice to the complete mutation analysis procedure, from flank PCR of genomic DNA to sequencing of the fragments S1–S4.

Statistical analysis

The overall frequency of *APC* mutations as well as the type of mutation was computed for all 665 cases with respect to age at diagnosis, gender and family history of CRC. Since tumour sub-localization was unknown for 11 patients, 654 patients could be analysed for different mutation types with regard to tumour sub-localization. Differences in mean age at diagnosis were evaluated pairwise between patients with wild-type *APC* and patients with *APC* mutations using the Mann–Whitney *U* test. The mean age at diagnosis of patients with an *APC* mutation, i.e. silent, missense or nonsense/frameshift mutations was compared with that of patients with wild-type *APC*. Differences in the categorical variables gender and family history of CRC, Dukes'stage, tumour differentiation and sub-localization between patients without and with *APC* mutations and type of mutation were evaluated using the χ^2-test. A *P*-value of 0.05 or less was considered to be statistically significant. All statistical procedures were performed with SPSS software (SPSS version 10.0, SPSS Chicago, IL).

Results

In the CRC cell lines HCT116, Colo205 and HT29, wild-type sequences were confirmed for the MCR of *APC*. Direct sequencing of the MCR of *APC* revealed a homozygous C > T substitution at codon 1367 in CaCo2 cells, a heterozygous C > T substitution at codon 1338 in SW480 cells and a deletion of C at codon 1430 in LOVO cells, as described previously (23). The effect of tissue processing as assessed in 10 specimens, showed that the mutation status of the MCR of *APC* for the paraffin-embedded block was identical to fresh unfixed tissue. The lowest level of detection was 5% mutant DNA in a background of wild-type DNA as found in three independent experiments. In 85% (61/72) of the fragments analysed for reproducibility assessment, the same *APC* mutation status was observed in duplicate experiments.

In 72% of tumours from CRC patients (479/665) a total of 978 mutations were observed in the MCR of the *APC* gene. The number of observed mutations per tumour ranged from one to eight mutations. In 215 tumours (45%) one mutation was found, in 137 tumours (29%) two mutations and in 127 tumours (27%) more than two mutations were observed. Of all mutations observed, 85% (833/978) were point mutations and 15% (145/978) were insertions and deletions (Table II). Point mutations were predominantly missense mutations (61%) and less frequently consisted of nonsense mutations (16%) or silent mutations (23%). Transitions were the most common type of point mutations (730/833), in large majority represented by C > T and G > A transitions ($n = 352$ and $n = 286$, respectively). Of C > T and G > A transitions 23% occurred at CpG dinucleotides. A polymorphism (G4497A), which does not result in an altered amino acid, was observed in 74% (493/665) of patients.

Eighty-seven per cent of frameshift mutations (126/145) were deletions (Table II). The length of the frameshift ranged from 1 to 5 bp for insertions and 1 to 49 bp for deletions. Generally, frameshift mutations would lead to serious alterations in the amino acid sequence downstream of the mutation

Table II. Number and type of mutations in APC MCR

Type of mutation	Mutation	Silent mutation[a]	Missense mutation[b]	Mutation leading to truncation[c]
Point mutation	833	192 (24%)	509 (61%)	132 (16%)
Transition				
C > T	352	63 (18%)	208 (59%)	81 (23%)
G > A	286	62 (22%)	223 (77%)	1 (0%)
A > G	63	24 (38%)	39 (62%)	0 (0%)
T > C	29	20 (69%)	9 (31%)	0 (0%)
Transversion				
A > C	2	0 (0%)	2 (100%)	0 (0%)
T > G	5	0 (0%)	3 (60%)	2 (40%)
A > T	11	4 (36%)	1 (9%)	6 (55%)
T > A	20	16 (80%)	3 (15%)	1 (5%)
C > A	9	1 (11%)	4 (44%)	4 (44%)
G > T	38	1 (3%)	3 (8%)	34 (89%)
C > G	11	1 (9%)	7 (64%)	3 (27%)
G > C	7	0 (0%)	7 (100%)	0 (0%)
Insertion[d]	19	–	–	18 (90%)
Deletion[e]	126	–	–	126 (100%)

All 978 mutations detected in tumours from 665 sporadic CRC patients are represented.
[a]The frequency of silent mutations is calculated by dividing the number of specific mutations (e.g. C > T transitions) resulting in silent mutations by the total number of that specific type of mutation.
[b]The frequency of missense mutations is calculated by dividing the number of specific mutations (e.g. C > T transitions) resulting in missense mutations by the total number of that specific type of mutation.
[c]The frequency of nonsense and frameshift mutations is calculated by dividing the number of specific mutations (e.g. C > T transitions) resulting in nonsense mutations by the total number of that specific type of mutation.
[d]One insertion spanned 3 bp and would not result in frameshift or truncation.
[e]One deletion spanned 2 bp and would result in frameshift but not truncation.

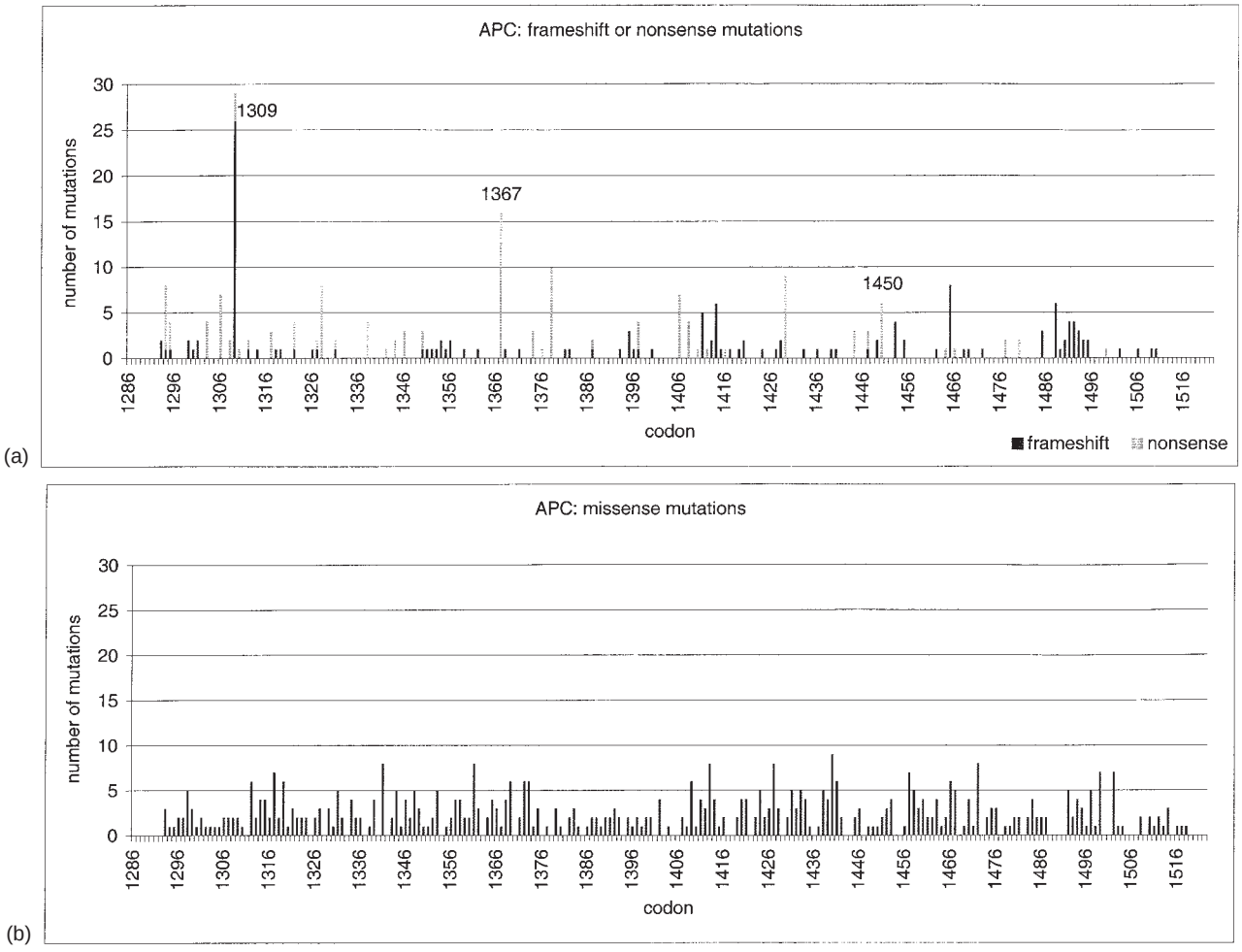

Fig. 1. Distribution and frequency of 143 frameshift mutations and 132 nonsense mutations (**a**) and 509 missense mutations (**b**) observed in tumour material from 665 sporadic CRC patients. The *x*-axis schematically represents the codons of the *APC* MCR.

and also result in the introduction of a stop codon. However, one insertion spanned 3 bp and would not result in frameshift or truncation.

In the distribution of the truncating mutations several hot spots could be observed (Figure 1a). The frameshift mutations seem to cluster in the regions at codons 1350–1356, 1411–1419, 1465, 1485–1495 and at codon 1309, where an AAAAG deletion was detected in 26 tumours. A high frequency of nonsense point mutations was detected at codons 1294, 1306, 1328, 1367, 1378, 1406, 1429 and 1450. The large number of missense mutations was distributed rather evenly throughout the MCR of *APC* and did not show distinct hot spots (Figure 1b).

The 479 patients that harboured mutations were divided into different groups on the basis of having a tumour with either a wild-type or a mutant *APC* with various types of gene mutations (Table III). In total, 52% (248/479) of the patients with a mutation in the MCR of *APC*, which corresponds to 37% of all patients (248/665), harboured at least one nonsense or frameshift mutation that would result in a truncated APC protein. Of patients with a truncating mutation, 59% (145/247) had more than one mutation leading to a change in the APC protein, i.e. another nonsense and frameshift mutation ($n = 27$) or missense mutation ($n = 118$). Forty per cent of the patients (191/479) with a mutation in the MCR, corresponding to 29% of all patients (191/665), harboured at least one missense mutation and no nonsense or frameshift mutations. Eighteen per cent of patients (84/479) had two or more missense mutations. Of all patients, 6% (40/665) had one or more silent mutations and no other type of mutation, and six of these patients had two silent mutations (Table III).

The mean age at diagnosis was lower for patients with mutation(s) in *APC* as compared with patients with wild-type *APC* (67.66 ± 4.21 versus 68.75 ± 4.32, respectively, $P = 0.002$) (Table III). Detailed analysis of subgroups of mutations revealed that patients with silent mutations ($P = 0.008$), missense mutations ($P = 0.065$) and nonsense or frameshift mutations ($P = 0.002$) were relatively younger when diagnosed with a colorectal tumour as compared with patients with wild-type *APC* (Table III). No differences were observed in the occurrence of *APC* mutations with respect to gender and family history of CRC ($P = 0.630$ and $P = 0.364$, respectively) (Table III). No statistically significant differences in the occurrence and type of *APC* mutations with respect to Dukes' stage and tumour differentiation were observed (data not shown).

No statistically significant overall difference between patients without and with any *APC* mutation was observed with respect to tumour sub-localization. However, when considering specific types of mutations, patients with rectosigmoid and rectal tumours had a relatively higher frequency of

Table III. Characteristics of 665 CRC patients without an APC mutation or with at least one silent mutation, missense mutation or nonsense mutation

	Total CRC patients	*APC* status		Type of *APC* mutation		
		Wild-type[a]	Mutation[b,c]	Silent mutation[c,d]	Missense mutation[c,e]	Nonsense or frameshift mutation[c,f]
Number of patients	665	186	479	40	191	248
Age at diagnosis (mean ± SD)	67.96 ± 4.26	68.75 ± 4.32	67.66 ± 4.21	66.85 ± 4.21	67.97 ± 4.31	67.55 ± 4.12
P-value[g]			0.002	0.01	0.07	0.002
Gender (male)	371	101 (54%)	270 (56%)	16 (39%)	112 (59%)	142 (57%)
P-value[g]			0.63	0.10	0.40	0.54
Family history of CRC (yes)	64	21 (11%)	43 (9%)	4 (10%)	16 (8%)	23 (9%)
P-value[g]			0.36	0.81	0.34	0.49
Sub-localization[h]	654	183	472	39	190	242
Proximal colon	223	71 (32%)	152 (68%)	15 (7%)	68 (31%)	69 (31%)
Distal colon	206	56 (27%)	150 (73%)	12 (6%)	72 (35%)	66 (32%)
Rectosigmoid	74	18 (24%)	56 (76%)	3 (4%)	14 (19%)	39 (53%)
Rectum	151	38 (25%)	113 (75%)	9 (6%)	36 (24%)	68 (45%)
P-value[g]			0.42	0.97	0.45	0.03

[a]Patients without any mutations.
[b]Patients with at least one mutation.
[c]The frequency of patients with a silent, missense or nonsense/frameshift mutation per sub-localization was calculated by dividing the number of patients per mutation status group (e.g. silent mutation) by the total number of CRC patients.
[d]Patients with at least one silent mutation, but without any missense or nonsense or frameshift mutations.
[e]Patients with at least one missense mutation, but without any nonsense or frameshift mutations.
[f]Patients with at least one nonsense or frameshift mutation.
[g]*P*-values were calculated for pairwise comparisons of distribution of sub-localization for patients with an *APC* mutation, with a silent mutation, with a missense mutation or with a nonsense/frameshift mutation to patients with wild-type *APC*.
[h]Information on tumour sub-localization was available for 654 patients.

Table IV. Characterization of most common types of truncating mutations according to sub-localization of the tumour in 654 sporadic CRC patients

	Total CRC patients	Wild-type APC[a]	Nonsense mutation		Frameshift[a,b,c]
			C > T transition[a,c,d]	G > T transversion[a,b,c,d]	
Sub-localization	654	183	75	33	138
Proximal colon	223	71 (32%)	15 (7%)	12 (5%)	42 (19%)
Distal colon	206	56 (27%)	25 (12%)	7 (3%)	33 (16%)
Rectosigmoid	74	18 (24%)	16 (22%)	8 (11%)	17 (23%)
Rectum	151	38 (25%)	19 (13%)	6 (4%)	46 (30%)
P-value[e]			0.01	0.12[f]	0.05

[a]For five patients frameshift as well as G > T transversions were found. These patients were included in both groups.
[b]For 13 patients frameshift as well as C > T transitions were found. These patients were included in both groups.
[c]The frequency of patients with wild-type *APC*, C > T transitions, G > T transversions or frameshift mutations per tumour sub-localization was calculated by dividing the number of patients per mutation group (e.g. C > T transition) by the total number of CRC patients.
[d]For one patient a C > T transition and a G > T transversion was found. This patient was included in both groups.
[e]*P*-values were calculated for pairwise comparisons of distribution of sub-localization for patients with at least one C > T transition ($n = 75$), patients with at least one G > T transversion ($n = 33$) or those with at least one frameshift mutation ($n = 137$) to patients with wild-type *APC*.
[f]*P*-value could not be interpreted due to lack of sufficient patients in respective groups.

truncating *APC* mutations as compared with patients with a proximal or distal colon tumour (53 and 45% versus 31 and 32%, respectively, $P = 0.029$) (Table III). Most mutations that would lead to a truncated APC protein are caused by C > T transitions, G > T transversions and frameshift mutations. Patients with proximal colon tumours have relatively less C > T nonsense mutations than patients with a distal colon, rectosigmoid and rectal tumour (7 versus 12%, 22 and 13%, respectively, $P = 0.009$) (Table IV). Patients with rectosigmoid and rectal tumours have a relatively higher frequency of truncating *APC* mutations as compared with patients with a proximal or distal colon tumour (23 and 30% versus 19 and 16%, respectively, $P = 0.045$) (Table IV). No statistically significant difference in the occurrence of G > T nonsense mutations was observed between patients with tumours at the different sub-localizations (Table IV). With regard to distribution of all C:G > T:A transitions over the different sub-localizations, irrespective of their putative effect on the APC protein, these seemed to occur relatively more frequently in distal colon, rectosigmoid and rectum as compared with the proximal colon, but this was not statistically significant (data not shown).

Discussion

The occurrence and type of somatic mutations in the MCR of the *APC* gene was evaluated in 665 tumour samples from an unselected, well-defined group of incident CRC patients from The Netherlands (age at diagnosis between 57 and 67 years). Seventy-two per cent of patients (479/665) harboured a total

of 978 mutations in the *APC* MCR. In particular, we observed a large number ($n = 508$) of missense point mutations. Mutations that would lead to protein truncation were found in 37% of patients (248/665). Patients with rectosigmoid and rectum tumours relatively more frequently harboured C > T nonsense mutations and deletions and insertions that would lead to protein truncation as compared with patients with proximal and distal colon tumours.

In this study, DNA from archival tumour tissue was used. As this formalin-fixed, paraffin-embedded tissue contains highly fragmented genomic DNA, gene analysis can be difficult. The analysis of *APC* mutations is based on nested amplification and direct sequencing of purified PCR fragments, a highly sensitive and specific detection method. Using this method on archival tissue yields valid results, as shown in the identical mutations status of fresh tissue and paraffin-embedded tissue. Moreover, most mutations leading to truncation of the APC protein observed in this study were also reported in an APC database (http://www.umd.necker.fr:2008/).

As a result of fragmentation of DNA from archival sources, a protein truncation test or similar methods are not feasible, as these techniques require fully intact DNA strands. In other studies, protein truncation test (7), *in vitro* synthesized protein/*in vitro* transcription and translation assay (19,24) were used, thereby selectively identifying truncating mutations. Although these methods provide conclusive information on the functional aberrations in *APC*, i.e. truncating mutations, these do not yield information on the occurrence of other than truncating mutations. Indeed, the large number of mutations detected in our study is mainly due to the high prevalence of other than truncating mutations.

Techniques used for mutation screening such as single-strand conformational polymorphism (8,16–18,21,31), RNase protection assay (7) or denaturing gradient gel electrophoresis (31) show differences in sensitivity and/or specificity of mutation detection. In particular, the interpretation of single-strand conformational polymorphism assays is difficult and its sensitivity is relatively low (32). Omission of a screening assay prior to sequence analysis may therefore partially explain the large number of mutations found in this study.

We observed multiple mutations in 55% of adenocarcinomas, ranging from two to eight mutations per tumour. Possibly, this reflects the occurrence of mutations in both alleles of the *APC* locus. Since *APC* is a tumour suppressor gene, more than one inactivating mutation would be necessary to result in loss of APC function in the cell. However, we are unable to distinguish different alleles in our analyses. Another explanation for multiple mutations within a tumour may be that it is a reflection of tumour heterogeneity. In a recent study of advanced colorectal tumours, heterogeneity in the mutational status of *p53* and *K-ras* within the same tumour was demonstrated (33). We performed macrodissection on the samples that were analysed, as a result of which we may have included different subclones of a tumour that possibly harbour different mutations.

Another plausible cause of multiple mutations is a defect in the DNA repair systems. Inherited variants of the base excision repair gene *MYH* were found to give rise to G > T and C > A transversions in a family that is affected with multiple colorectal adenomas and carcinoma (34). Recently, these inherited variants of *MYH* have also been observed in a population-based series of CRC patients (35), suggesting that a proportion of the 46 patients that harboured a G > T or C > A transversion in our study may be due to inhereted *MYH* mutations. Defects in the mismatch repair genes *hMLH1* and *hMSH2*, resulting in mismatch repair deficiency is found in hereditary non-polyposis coli and is also found in ~15% of sporadic colorectal tumours. However, reported results are inconsistent as to whether mismatch repair deficiency is an alternative pathway in colorectal tumours with intact APC function (36,37) or that it independently gives rise to an increased frequency of *APC* mutations (38–40).

The majority of observed mutations were point mutations, 61% of which give rise to substitution of one amino acid. The relevance of missense mutations in *APC* is not yet understood. It is unclear which of these changes would affect the structure and function of the APC protein and whether such an alteration would lead to selective advantage of tumour cells and tumour growth. Intriguingly, 52% of patients with truncating mutations also had one or more concurrent missense mutation(s). If the occurrence of these missense mutations is the reflection of a biallelic event, this may indicate involvement of *APC* missense mutations in colorectal tumourigenesis.

Twenty-three per cent of the point mutations would not lead to protein truncation or an altered amino acid. In addition, a polymorphism (G4497A), which does not result in an altered amino acid was observed in 74% (493/665) of patients. This polymorphism has also been described recently in a series of adenoma samples from Dutch patients (31), which suggests that this polymorphism represents a wild-type DNA sequence in *APC* in the Dutch population.

Considering all mutations that are detected in the MCR, only 28% (276/978) would lead to a truncated APC protein, half of which are represented by point mutations. This is in contrast with previous reports that up to 98% of mutations would lead to a truncated APC protein, and only one-third of these are caused by point mutations (25).

Only 37% of patients (247/665) harboured mutations that would lead to a truncated APC protein. Considering the general idea that most colorectal tumours follow a molecular pathway involving APC, our observed frequency seems low. It is, however, in keeping with the frequency of 34% truncating mutations found in the MCR in another Dutch population-based case-control study (21). The MCR spans several 20 aa repeats, which form the β-catenin binding sites and therefore the MCR represents a functionally important domain of the *APC* gene. According to several reports 68–77% of the somatic mutations in *APC* are found in the MCR, which represents < 10% of the *APC* coding region (5,7,8). We are unable to make any predictions of the occurrence of mutations in the complete *APC* gene, since our mutation analysis was restricted to the MCR of *APC*.

Other explanations for the relatively low frequency of truncating mutations of *APC* in the colorectal tumours observed here, are that these tumours may have arisen through other genetic or epi-genetic events. APC is a key player in the Wnt pathway. Inactivating mutations that cause loss of β-catenin binding and degradation sites on the APC protein, would lead to increased levels of intra-nuclear β-catenin (9,22). However, it has also been shown that activating mutations in the *CTNNB1* gene can lead to stabilization and accumulation of the β-catenin protein (41,42), thereby circumventing the necessity for mutations in *APC* to activate the Wnt pathway. Hypermethylation of the promotor region of *APC* constitutes an alternative mechanism for gene inactivation. This was found to occur in 18% of primary sporadic colorectal carcinomas (43), and in 39% of a sample of 122 CRC patients from

our study, although these cases were selected on high and low methyl donor intake (44).

FAP tumours generally arise at a young age through a germline mutation in the *APC* gene. Our *APC* gene mutation analysis is based on a relatively large series of unselected, incident CRC patients, who were between 55 and 69 years of age at baseline and have developed a first colorectal tumour since. This strongly suggests that the mutations observed in this study are acquired and therefore of somatic nature. Moreover, no significant differences in family history of CRC between patients without and with *APC* mutations were observed, which also argues against germline mutations. The observation that there were no statistically significant differences in occurrence and type of *APC* mutations with respect to Dukes' stage and tumour differentiation support the hypothesis that *APC* gene mutation is an early event in colorectal tumourigenesis.

The differences in the occurrence of specific types of mutations in colon versus rectosigmoid and rectum cancer shown here, suggests a different aetiology of tumourigenesis in the different sub-localizations and it may also reflect the exogenous origin of mutations. This is supported by a recent report of *K-ras* mutations in CRC tissue of patients from our study, in which mutations in *K-ras* were also more frequently observed in rectum tumours as compared with rectosigmoid and colon tumours (29). The data with respect to the rectosigmoid have to be regarded with some caution as the rectosigmoid is a more clinically applied term rather than an anatomically defined transitional zone between colon and rectum (29). When rectosigmoid tumours were excluded from the analyses, both frameshift and C > T mutations that would result in protein truncation occurred relatively more frequently in the rectum as compared with proximal and distal colon. However, the asymmetry of C > T nonsense mutations with regard to tumour sub-localization was less pronounced.

In conclusion, a large number of mutations was found in the MCR of the *APC* gene, in particular, we observed many missense point mutations, more than reported previously. Missense mutations need further exploring with regard to their role in APC function and colorectal tumourigenesis. Patients with a rectosigmoid or rectum tumour relatively more frequently have a truncating C > T transition or frameshift mutation as compared with patients with a proximal or distal colon tumour. The pattern of *APC* mutations observed in the different sub-localizations of the colorectal tract suggests a different aetiology for tumour site within the large bowel.

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